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Apoptosis and Cell Proliferation in Medullary Carcinoma of the Breast: A Comparative Study Between Medullary and Non-Medullary Carcinoma Using the TUNEL Method and Immunohistochemistry

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Background and Objectives: Medullary carcinoma of the breast has generally been considered to result in better prognosis than ordinary invasive ductal carcinoma, which would seem to be discrepant when one considers its anaplastic histology and high mitotic rate. We attempted to elucidate the prognostic implications of apoptosis and cell proliferation in medullary carcinoma of the breast.

Methods: Formalin-fixed, paraffin-embedded specimens of 50 cases of typical medullary carcinoma (MC) of the breast and those of 50 control cases of non-medullary invasive ductal carcinoma (N-MC), which were matched to the MC cases in both age and TNM classification, were investigated utilizing the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method and immunohistochemistry for p53, bcl-2, and Ki-67.

Results: Mean values of the apoptotic index (AI), the proliferative index (PI), and the ratio of AI to PI (AI/PI) were significantly higher in MC than in N-MC ($P < 0.0001$). MC exhibited significantly lower positivity for bcl-2 than N-MC ($P = 0.00003$), while there was no significant difference in p53 positivity between MC and N-MC.

Conclusions: A high frequency of apoptosis may be related to a favorable prognosis in MC, even though it demonstrates a high proliferative activity, exhibiting a rapid cell turnover.

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KEY WORDS: **medullary carcinoma of the breast; apoptosis; proliferative activity; TUNEL method; immunohistochemistry; p53; bcl-2**

INTRODUCTION

It has generally been considered that medullary carcinoma (MC) of the breast results in better prognosis than ordinary invasive ductal carcinoma [1–5]. Its excellent prognosis has been thought to be related to its characteristic histologic features such as substantial mononuclear infiltrates [6,7] and a syncytial growth pattern [8], although the carcinoma cells are characterized by poor differentiation and have a high mitotic rate [9–11], which would seem to conversely affect the prognosis. This has continued to be a biological paradox intrinsic to MC of the breast.

It could be thought that neoplastic growth is brought

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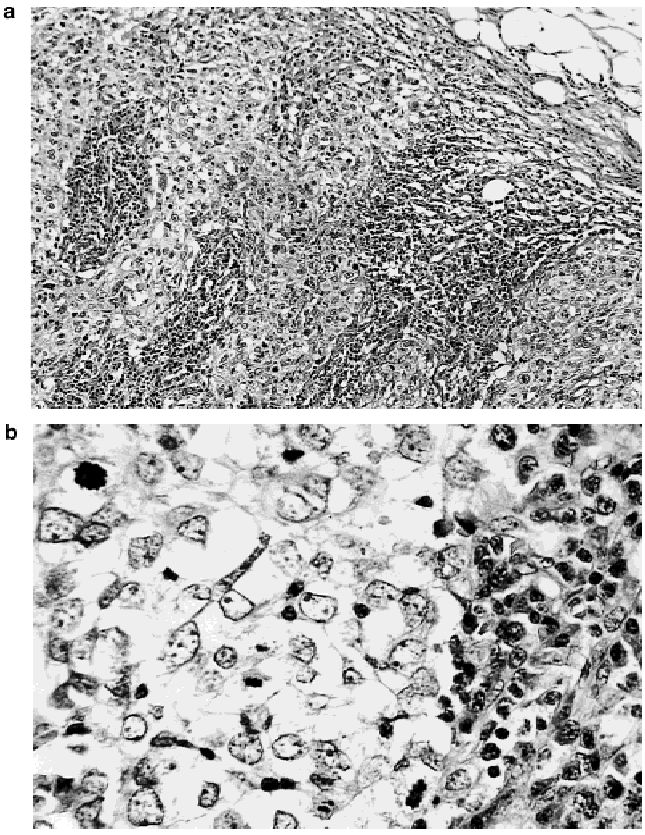

Fig. 1. Histologic view of MC of the breast. **(a)** Low-power view (H&E, ×100). **(b)** High-power view (H&E, ×400).

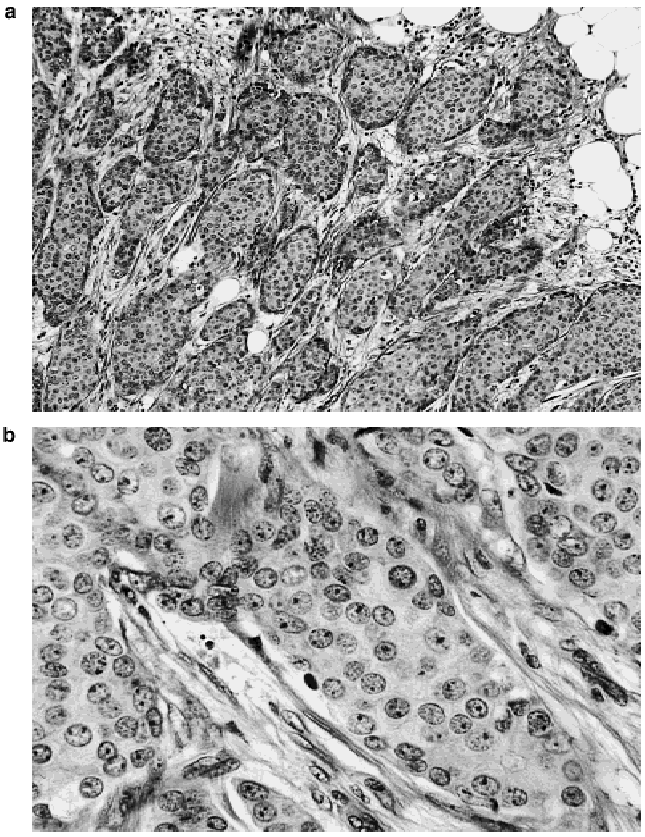

Fig. 2. Histologic view of N-MC of the breast. **(a)** Low-power view (H&E, ×100). **(b)** High-power view (H&E, ×400).

about by evasion of the cell kinetic homeostasis. Disorders of cell accumulation could be considered to develop when the rate of cell proliferation exceeds that of cell death [12]. It has been reported that apoptotic cell death is involved not only in physiologic processes but also in pathologic processes, including cell proliferation and cell turnover [13–18]. Tumor growth should thus be analyzed on the basis of both cell proliferation and apoptosis.

Gavrieli et al. [19] introduced the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method to detect apoptotic cells in formalin-fixed, paraffin-embedded sections [19]. Therefore, we attempted to clarify the prognostic implications of apoptosis and cell proliferation in MC of the breast, utilizing the TUNEL method and immunohistochemistry for Ki-67 and the apoptosis-related antigens p53 and bcl-2.

MATERIALS AND METHODS

Materials

We studied 50 cases of typical MC of the breast (Fig. 1a,b), selected strictly according to the criteria proposed by Ridolfi et al. [4], in that they met all of the following: (1) predominantly syncytial growth pattern (>75%); (2) microscopically completely circumscribed; (3) no intraductal component; (4) moderate to marked diffuse mononuclear stromal infiltrates; (5) high nuclear grade of tumor cells; (6) absence of microglandular structures.

All 50 MC patients were women and ranged in age from 19 to 82 years (mean: 53 years). All the patients underwent either modified or radical mastectomy with regional lymph node dissection. The present series included neither cases of quadrantectomy nor of lumpectomy. According to the TNM staging system, 16 patients were stage I, 31 were stage II, and 3 were stage III.

For comparison, 50 cases of non-medullary invasive ductal carcinoma (N-MC), which exhibited in particular an expansive growth with an almost circumscribed margin and which were composed of microscopically solid nests (in the sense that glandular or papillary structures are hardly present within the nests) of an alveolar or sheet pattern with or without mononuclear infiltrates (Fig. 2a,b), were employed, with the intentional exclusion of scirrhous carcinoma, the microscopic features of which are quite different from those of MC, although this type accounts for the majority of the ordinary invasive ductal carcinomas. Moreover, the control cases were matched to the MC cases in both age and TNM classification.

All of the representative formalin-fixed, paraffin-embedded blocks of these 100 breast carcinomas dating

from the period of 1976 to 1994, inclusive, were supplied by the Second Department of Pathology, Kyushu University (Fukuoka, Japan), and its affiliated institutes including the Department of Pathology, Kitakyushu Municipal Medical Centre (Kitakyushu, Japan). Serial sections (4 μm thick) were stained with hematoxylin-eosin (H&E), and these were then analyzed by the TUNEL procedure and immunohistochemistry for Ki-67 and the apoptosis-related antigens p53 and bcl-2.

TUNEL Technique

DNA breaks were detected in situ by nick end-labeling with the TUNEL method, which is based on the specific binding of TdT to the 3′-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer [19]. For this purpose, the ApopTag™Plus In Situ Apoptosis Detection Kit-Peroxidase (Oncor, Gaithersburg, MD) was utilized. Briefly, after routine deparaffinization, rehydration, and blocking of endogenous peroxidase with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature (RT), the tissue sections were digested with 20 μg/ml proteinase K (Sigma, St. Louis, MO) for 15 min at RT. After they were washed in distilled water, Equilibration Buffer (ApopTag Kit) was applied to the sections for 60 sec at RT, followed by incubation with Working Strength TdT Enzyme (ApopTag Kit), and then they were covered with a coverslip in a humidified chamber for 60 min at 37°C. The reaction was terminated in prewarmed Working Strength Stop/Wash Buffer (ApopTag Kit) for 30 min at 37°C. After being washed in phosphate-buffered saline, the sections were covered with Anti-Digoxigenin-Peroxidase (ApopTag Kit) for 30 min at RT, followed by color development with 3,3′-diaminobenzidine (DAB)-H_2O_2 solution. The sections were counterstained with hematoxylin.

To confirm the staining specificity, the TUNEL procedure was modified as follows: for the positive control, control slides (ApopTag Kit) were stained as described above. The negative control sections were obtained by substituting distilled water for TdT.

Immunohistochemistry

Immunohistochemical staining was carried out by the streptavidin-biotin-peroxidase complex method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) applying monoclonal antibodies raised against Ki-67 (MIB-1, dilution 1:100; Immunotech, Marseille, France), p53 (Pab 1801, dilution 1:100; Oncogene Research Products, Cambridge, MA), and bcl-2 (clone 124, dilution 1:1,000; DAKO, Glostrup, Denmark) after antigen retrieval with microwave heating (citrate buffer, 30 min; phosphate-buffered saline, 10 min; citrate buffer, 30 min; respectively) using an H2800 Microwave Processor (Energy Beam Sciences, Agawan, MA) at 800 W. Sections were visualized with DAB and counterstained with hematoxylin. The positive controls used were as follows: non-neoplastic gastric mucosa, which inevitably possesses proliferative zones, for MIB-1; a specimen of the gastric carcinoma, in which the point mutation of the p53 gene was confirmed by polymerase chain reaction-single-strand conformation polymorphism analysis, for p53; and tissue from a normal lymph node for bcl-2. The negative controls consisted of substituting mouse normal serum for the primary antibodies.

Labeling Index

The apoptotic index (AI) was defined as the percentage of TUNEL-positive cells relative to counted carcinoma cells in the clearly labeled areas, as determined by scanning at a low magnification. The AI was determined by counting at least 1,000 cells in the selected fields at ×400 magnification. Multiple fields were necessary to obtain more than 1,000 cells for each case. Serial H&E sections were observed in order to avoid miscounting necrotic cells as far as possible.

The proliferative index (PI) was obtained as the percentage of Ki-67-immunopositive nuclei. The PI was determined by counting more than 1,000 carcinoma cells in the same manner as described for the AI.

The ratio of AI to PI (AI/PI) was also calculated as an indicator of the rate of cell loss relative to that of cell proliferation [20].

Immunostaining Assessment

p53 immunoreactivity was defined as positive when distinct nuclear staining was recognized in at least 10% of the carcinoma cells, since most published reports have employed that as the cutoff level. Cases with less than 10% positive cells were regarded as negative.

bcl-2 immunoreactivity was evaluated by a semiquantitative method, which takes account of the distribution of positive cells and the intensity of cytoplasmic staining, and was recorded as positive when the carcinoma cells showed diffuse and unequivocally strong cytoplasmic immunoreaction. Cases with only focal or equivocally weak staining were regarded as negative.

Statistical Analysis

Statistical analysis was performed by using the Mann-Whitney *U*-test. Cumulative survival curves were estimated by the Kaplan-Meier method and the differences were analyzed with both the generalized Wilcoxon test and the log-rank test. A probability value below 0.05 ($P < 0.05$) was considered statistically significant.

RESULTS

Histologic Findings

Provided that the cases had been selected strictly according to the criteria of Ridolfi et al. [4] for typical MC, the histologic variance should have been very slight.

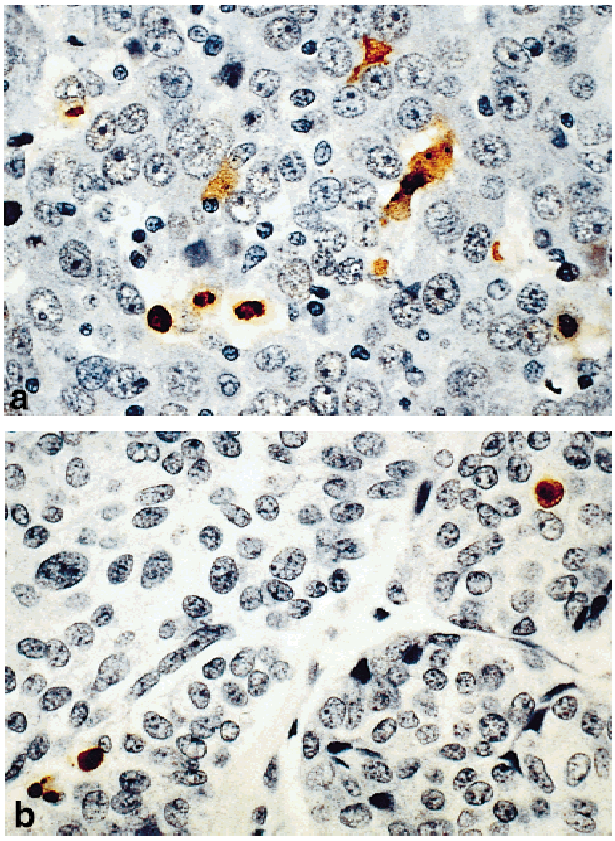

Fig. 3. **(a)** Several apoptotic cells labeled by the TUNEL method in MC (×400). **(b)** A few apoptotic cells in N-MC (×400).

However, of 50 MC cases, only 1 case revealed squamoid configuration with multinucleated giant cells and 6 cases exhibited substantial necrosis. Most of the MC cases showed more apoptotic cells, as evidenced by apoptotic bodies, than the N-MC cases, even in H&E-stained sections.

AI and PI

TUNEL signals were detectable within the nuclei of the carcinoma cells. An intense TUNEL signal was observed even in ordinary, non-pyknotic nuclei of carcinoma cells and occasionally in nuclear fragments that corresponded to apoptotic bodies. In most cases, the TUNEL method demonstrated that MC (Fig. 3a) had more apoptotic cells than N-MC (Fig. 3b). Moreover, in most cases, MC showed more positive nuclei for Ki-67 (Fig. 4a) than N-MC. The overall results, including AI and PI, are shown in Table I. AIs ranged from 0.3 to 4.4 (mean ± standard deviation: 2.1 ± 1.1) in MC and from 0 to 0.9 (0.2 ± 0.3) in N-MC. The mean AI of MC was significantly higher than that of N-MC ($P < 0.0001$). PIs ranged from 0 to 80.2 (44.2 ± 21.6) in MC and from 3.3 to 48.7 (18.8 ± 12.5) in N-MC. The mean PI of MC was significantly higher than that of N-MC ($P < 0.0001$). The mean AI/PI ratio for MC was significantly higher than

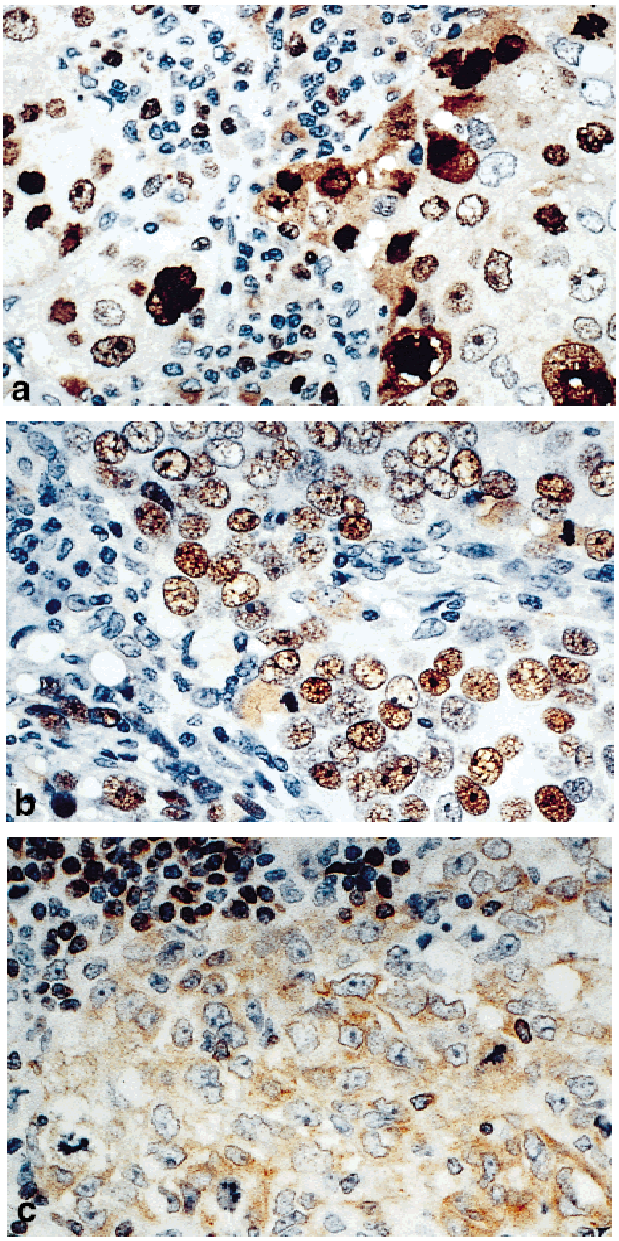

Fig. 4. **(a)** Nuclear immunostaining for Ki-67 in MC (×400). **(b)** Nuclear immunostaining for p53 in MC (×400). **(c)** Cytoplasmic immunostaining for bcl-2 in MC (×400).

TABLE I. AI and PI in MC and N-MC†

	AI	PI	AI/PI
MC	2.1 ± 1.1*	44.2 ± 21.6**	5.9 ± 4.0***
N-MC	0.2 ± 0.3*	18.8 ± 12.5**	1.1 ± 1.4***

†Values are means ± standard deviations.
*, **, ***$P < 0.0001$.

TABLE II. p53 and bcl-2 Positivity in MC and N-MC

Type	p53(+)/bcl-2(+)	p53(+)/bcl-2(−)	p53(−)/bcl-2(+)	p53(−)/bcl-2(−)
MC	5	18	2	25
N-MC	11	11	21	7

TABLE III. p53 Positivity in MC and N-MC and Its Correlation With AIs and PIs†

Type	p53	No. (%)	AI	PI	AI/PI
MC	(+)	23/50 (46)	2.7 ± 1.3***	46.2 ± 23.4	6.2 ± 3.3
	(−)	27/50 (54)	1.9 ± 1.1***	42.5 ± 20.3	4.9 ± 3.6
N-MC	(+)	22/50 (44)	0.3 ± 0.3*	24.6 ± 12.9**	1.6 ± 1.6****
	(−)	28/50 (56)	0.1 ± 0.2*	14.3 ± 10.4**	0.8 ± 0.1****

†Indices are means ± standard deviations.
*, **$P < 0.01$.
***$P = 0.023$; ****$P < 0.05$.

TABLE IV. bcl-2 Positivity in MC and N-MC and Its Correlation With AIs and PIs†

Type	bcl-2	No. (%)	AI	PI	AI/PI
MC	(+)	8/50 (16)*	1.4 ± 1.0**	36.5 ± 28.2	7.6 ± 6.0
	(−)	42/50 (84)	2.3 ± 1.1**	45.6 ± 20.4	5.3 ± 3.5
N-MC	(+)	32/50 (64)*	0.2 ± 0.2***	18.4 ± 13.5	0.8 ± 1.0****
	(−)	18/50 (36)	0.4 ± 0.4***	19.6 ± 11.0	1.8 ± 1.9****

†Indices are means ± standard deviations.
*$P = 0.00003$.
$P = 0.037$; *, ****$P < 0.05$.

that for N-MC ($P < 0.0001$). Furthermore, there were mutually significant differences between AI, PI, and AI/PI in MC and N-MC, respectively ($P < 0.01$).

Apoptosis-Related Antigens

p53 and bcl-2 positivity in MC and N-MC is summarized in Table II. The relationships between p53 and bcl-2 positivity and AI and PI are shown in Tables III and IV. Twenty-three of 50 MCs (46%) exhibited nuclear positivity for p53 (Fig. 4b). Twenty-two of 50 N-MCs (44%) were p53-positive. No significant difference was observed regarding p53 positivity between MC and N-MC ($P = 1.0$). Concerning bcl-2, only 8 of 50 MCs (16%) demonstrated a positive cytoplasmic immunohistochemical reaction (Fig. 4c), whereas 32 of 50 N-MCs (64%) were positive. The difference was statistically significant ($P = 0.00003$). Moreover, there was no significant association between p53 and bcl-2 positivity in MC and N-MC, respectively.

In MC, the mean AI for p53-positive carcinoma was significantly higher than that for p53-negative carcinoma ($P = 0.023$), while the mean AI for bcl-2-negative carcinoma was significantly higher than that for bcl-2-positive carcinoma ($P = 0.037$), although the PI and the AI/PI ratio demonstrated no statistically significant relationship to p53 or bcl-2 positivity.

In N-MC, the mean values of AI, PI, and AI/PI for p53-positive carcinoma were significantly higher than those for p53-negative carcinoma ($P < 0.01$, 0.01, 0.05, respectively). The mean values of AI and AI/PI for bcl-2-negative carcinoma were significantly higher than those for bcl-2-positive carcinoma ($P < 0.05$), with PI exhibiting no statistically significant correlation to bcl-2 positivity.

In addition, in both MC and N-MC, no significant relationships were reciprocally detected between clinicopathologic parameters such as patients' age, tumor size, or regional lymph node status, and the staining results or indices obtained in this study: p53, bcl-2, AI, PI, and AI/PI.

Prognosis

Survival information was available for 44 of the 50 MC cases and for all of the 50 N-MC cases. The follow-up periods of the MC patients ranged from 30 to 250 months (mean: 92 months) while those of the N-MC patients ranged from 9 to 151 months (mean: 68 months). Two of the 44 MC patients and 5 of the 50 N-MC pa-

tients died of the disease during the follow-up period. The 5- and 10-year survival rates for the MC cases were 97.5% and 93.1%, respectively, and for the N-MC cases were 90.9% and 86.4%, respectively. The survival probability thus appeared to be somewhat higher for the former than it was for the latter, although the difference was not statistically significant.

bcl-2-positive N-MC showed a lower survival probability than bcl-2-negative N-MC ($P < 0.01$). However, no significant correlation was produced either in MC or in N-MC regarding other clinicopathologic variables, including tumor size and p53 positivity.

DISCUSSION

It has generally been accepted that MC of the breast results in better prognosis than ordinary invasive ductal carcinoma [1–5], even though it exhibits poorly differentiated nuclei and a high mitotic rate [9–11]. Its favorable prognosis has been thought to be related to its particular histological features, namely, a syncytial growth pattern [8] and substantial mononuclear infiltrates within the stroma [6,7].

It is known in general that neoplastic cells have higher proliferative activity than normal cells [21,22]. Neoplastic growth may be caused or promoted by the inhibition of cell death [23]. The net growth of a tumor is supposed to depend upon the balance between cell proliferation and cell loss [18,24–26]. It is therefore desirable that the tumor growth should be analyzed on the basis of not only cell proliferation but also cell loss, although the majority of previous studies have focused chiefly on cell proliferation.

In the present study, we investigated apoptosis and proliferative activity in MC of the breast using the TUNEL method and immunohistochemistry for Ki-67 antigen and the apoptosis-related antigens p53 and bcl-2. The TUNEL technique provides a useful method for detecting apoptotic cells in formalin-fixed, paraffin-embedded sections [19]. Ki-67 antigen is a nuclear antigen expressed in all phases of the cell cycle except for G0, while the Ki-67-labeling index has been reported as providing a reliable and reproducible marker of proliferative activity [27,28]. Apoptosis might be regulated by tumor-suppressor genes and/or proto-oncogenes such as p53 [29], bcl-2 [30,31], and c-myc [32]. The most important function of wild-type p53 protein is the maintenance of genome integrity [33] by preventing a cell with DNA damage from entering the cell cycle [34]. Subsequently, DNA repair can take place, or apoptosis will be induced if the DNA damage is irreversible [35]. bcl-2 is a proto-oncogene that is involved in the regulation of cell death by inhibiting apoptosis in many cell systems in physiologic and neoplastic conditions [36].

In this research, the mean AI was significantly higher in MC than in N-MC. With regard to apoptosis-related antigens, MC revealed a significantly lower positivity for bcl-2 than N-MC, while there was no statistical significance in p53 positivity between MC and N-MC. Both in MC and in N-MC, the mean AI for p53-positive cases was significantly higher than that for p53-negative cases, while the mean AI for bcl-2-negative cases was significantly higher than that for bcl-2-positive cases, although no inverse correlation was found in the series between p53 and bcl-2, in contrast to that reported in several other studies [37–41]. Our data seem to suggest that apoptosis in the cases analyzed in this study is regulated by p53 and/or bcl-2. However, the regulation of apoptosis is complex and cannot simply be reduced to an assessment of p53 and/or bcl-2 [42]. The complete elucidation of these mechanisms remains an issue for further investigation.

In the current study, the mean values of the AI, the PI, and the AI/PI ratio were significantly higher in MC than in N-MC ($P < 0.0001$). These findings could indicate that MC undergoes a more rapid cell turnover than N-MC, while the high PI in MC could correspond to a high mitotic rate [11], which is supposed to be characteristic of MC. Recently, Jensen et al. [43] reported that MC revealed a high PI and an absence of the apoptosis-inhibitor protein bcl-2, which they postulated to be associated with the increased number of apoptotic cells. Moreover, they speculated that MC undergoes a high cell turnover and that increased apoptosis balancing the increased cell proliferation implies a favorable prognosis of MC. We also obtained similar results, although MC exhibited not absent but low positivity for bcl-2. Furthermore, using the TUNEL method, we were actually able to corroborate the finding that MC revealed a high incidence of apoptosis, which may play an important part in the prognosis of MC.

MC is accompanied by considerable mononuclear infiltrates, and it is this fact that is assumed to be related in some way to the excellent prognosis of MC [6,7]. However, we were unable to verify the association between apoptotic frequency and the intensity of the infiltrates, because each selected MC case was accompanied by not sparse or mild infiltrates, but by moderate to marked infiltrates (in accordance with the definition of MC), resulting in there being no possibility of comparisons according to intensity. Nevertheless, our results showed that MC demonstrated an increased occurrence of apoptosis. Some investigators have reported that cytokines alone or cytokines in conjunction with immune effector cells could induce apoptosis of tumor cells [44–46]. It is conceivable that the mononuclear infiltrates play some role in eventually inducing apoptosis of the carcinoma cells, however, the mechanism behind this remains largely unknown at present.

Survival analysis in this study yielded no significant difference between MC and N-MC, both showing excel-

lent prognosis with a 5-year survival rate of more than 90%. This arose naturally due to the selection of the control N-MC cases, which were matched to the MC cases in both age and TNM classification and from which scirrhous carcinoma had been excluded intentionally since we intended to compare the cases exhibiting microscopically solid growth pattern. It was also partly due to the fact that the number of deceased patients was too few for comparison in the series, and because advanced cases were relatively small in number among the present cases. Further investigation based on a larger number of cases should provide more accurate prognostic information.

CONCLUSIONS

MC of the breast and N-MC were investigated regarding apoptosis and cell proliferation utilizing the TUNEL method and immunohistochemistry, respectively. A high frequency of apoptosis may be related to a favorable prognosis in MC of the breast, even though it demonstrates a high proliferative activity, exhibiting a rapid cell turnover.

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